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**Feeding system and lactation stage affect the donkey milk fatty acid composition and fat-soluble vitamin composition**

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Short title: The feeding system affects the composition of donkey milk

#### **Abstract**

Donkey milk is considered a functional food for sensitive consumers, such as children allergic to cow milk. No information is available regarding the effect of the feeding system on the composition of donkey milk according to the feeding strategies adopted on commercial farms. The study was aimed at evaluating the effect of the feeding system and stage of lactation on the donkey milk gross composition, fat soluble vitamins (retinol,  $\alpha$ -tocopherol) and fatty acid (FA). Individual milk was sampled from lactating jennies (n=53) on six farms located in North West Italy. The performance of lactating jennies, the herd characteristics, milking management and feeding strategies were recorded at each milk sampling. A greater effect of the farming system and a limited effect of the lactation stage on the milk composition were observed. The gross composition of the milk, and the fat-soluble vitamin content differed according to the feeding system. A higher milk fat content corresponded to a higher fresh herbage proportion in the diet. The highest polyunsaturated FA (PUFA) content in the milk was observed for the animals fed on only forage diets. The animals that were fed just pasture produced the milk with the highest concentration of C18:1c9, C18:3n-3, n-3 FA, PUFA, retinol and  $\alpha$ -tocopherol, and the lowest concentrations of the FA less favorable for human health. The farms

that fed intermediate fresh herbage proportions in the diets showed intermediate concentrations of C18:3n-3 in the milk. Pasture feeding has been shown to improve the fat content and fat-soluble vitamin concentration of donkey milk and to move the FA composition to a more favorable profile for human nutrition, as already observed for ruminants.

**Keywords:** *Equus asinus*, Donkey milk, Lactation stage, Feeding system, Fatty acids.

## **Implications**

The present study has evaluated the effect of the feeding system and stage of lactation on the composition of donkey milk, considering data collected during a survey on dairy donkey farms in North West Italy. The results have shown that it is possible to move donkey milk composition to a more favorable profile for human nutrition, by means of feeding pasture to the lactating donkeys. These findings will be useful for dairy donkey breeders for improving the quality of donkey milk that is considered a functional food for sensitive consumers, such as children allergic to cow milk.

## Introduction

Donkey milk consumption is widespread in the Mediterranean area and, the EU production is estimated to be about 300 tons per year (Eurolactis, 2016, personal communication). The dairy donkey farms in the EU are mainly located in Italy, France, Spain and Belgium (Salimei and Fantuz, 2012). Clinical studies have indicated that donkey milk can be used successfully as an alternative to the available hypoallergenic formulas for infants suffering from cow milk protein allergy (Monti *et al.*, 2007). It has also recently been demonstrated *in vivo* that dietary supplementation with donkey and human milk is associated with a decrease in inflammatory status, and this decrease is in turn associated with an improvement in the lipid and glucose metabolism, compared to a diet with a cow milk supplementation (Trinchese *et al.*, 2015). The composition of donkey and human milk are similar, in terms of average total solid, crude protein, lactose and ash content. However, the fat content of donkey milk is lower than the fat content of human milk, as it is in the 0.3 to 1.2 g/100 mL range. This difference is associated with a low energy content (Salimei *et al.*, 2004; Medhammar *et al.*, 2012), which represents the main limit to its use in the nutrition of children allergic to cow milk protein, during the first year of life. However, the lipid fraction of donkey milk has shown a more favorable fatty acid (FA) composition than that of the milk fat of ruminants, as it is richer in polyunsaturated FA (PUFA) (Medhammar *et al.*, 2012). More in detail, donkey milk fat has shown higher C18:3n-3 and n-3 FA concentration, and a lower saturated FA (SFA) content than cow milk, as well as a lower n-6 to n-3 FA ratio (Medhammar *et al.*, 201). On the other hand, equid milk appears to have a lower fat-soluble vitamin content, that is, of  $\alpha$ -tocopherol and retinol, than ruminant milk (Gentili *et al.*, 2013; Álvarez *et al.*, 2015).

The variables that are significantly associated with changes in donkey milk composition are (1) the lactation stage; (2) daily rhythms; and (3) the interval between mechanical milkings (Salimei and Fantuz, 2012). However, feeding is also believed to play a relevant role in milk yield and composition, since nutrient absorption in equines precedes the ceco-colic fermentations of feeds (Doreau *et al.*, 2002). The feeding composition has been shown to be the main factor that affects the FA composition of milk in ruminants (Shingfield *et al.*, 2013; Coppa *et al.*, 2015a). In particular, pasture feeding increases in milk the concentrations of FA that are more favorable for human nutrition, such as C18:3n-3, n-3 FA and conjugated linoleic acids (CLA), and decreases the n6 to n3 ratio and the concentrations of FA less favorable for human nutrition, such as C14:0, C16:0, and SFA (Coppa *et al.*, 2012; Farruggia *et al.*, 2014). However, the effect of the feeding system on donkey milk composition has only been studied so far in experimental conditions for a restricted group of FA (Chiofalo *et al.*, 2005), and no information is available regarding the effect of the feeding system on the FA composition of donkey milk according to the feeding strategies adopted on commercial farms. Furthermore, changes in donkey milk fat-soluble vitamins, as a result of the feeding system, have never been investigated.

The aim of this study was to evaluate the effect of the feeding system and lactation stage on the milk composition of dairy asses, on the basis of observational data collected during a survey on six commercial farms located in North-West Italy.



## Materials and methods

### *Milk Sampling and Survey*

Individual milks were sampled (0.5 L) from 53 lactating jennies reared on six commercial farms located in the Piedmont Region, in North West Italy, during Spring 2014. The performance of the lactating jennies and herd characteristics (number of jennies, breed, DIM, milk yield, body condition scores (BCS), milking management, feeding strategies, forage type and conservation methods adopted were recorded at each milk sampling and characterized through a detailed on farm survey. The BCS were determined as described by Burden (2012), and body weight according to Pearson and Ouassat (2000). The farm characteristics, herd composition and diets of the jennies are reported in Table 1. The milk samples were immediately refrigerated, stored at -20°C and lyophilized within 72 h. The lyophilized samples were then stored at -20°C.

### *Milk Gross Composition Analyses*

The donkey milk samples were analyzed for fat, proteins, lactose and total solids contents. The fat content and protein content were assessed as described by Cavallarin *et al.*, (2015). The lactose content was determined by means of spectrophotometric absorbance at 340 nm (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA), according to the AOAC 984.15 Official Method (2005).

#### *Milk Fat-soluble Vitamin Analysis*

The retinol and  $\alpha$ -tocopherol in the milk samples were extracted according to the Kuhl *et al.* (2012), with some adaptations. The retinol and  $\alpha$ -tocopherol concentrations were quantified according to Prola *et al.* (2013), by means of a HPLC system (Dionex, Sunnyvale, CA, USA). The analytical column was an XTerra RP18 column (250-mm  $\times$  4.6-mm, 5  $\mu$ m particles) (Waters, Milford, MA).

A calibration curve was obtained with two determinations of six concentration levels of  $\alpha$ -tocopherol and retinol standard solutions (Sigma-Aldrich, St. Louis, MO) between 0.7 and 100  $\mu$ g/mL. The linearity was excellent ( $R^2 = 0.999$ ). Recovery experiments were performed by spiking blank donkey milk samples with retinol and with  $\alpha$ -tocopherol. The recoveries of the method were good, ranging from 91.1% to 96.8% (Table 3).

#### *Milk Fatty Acid Analysis*

Milk samples were analyzed for FA composition by gas chromatography (GC), as described by Coppa *et al.* (2015b). The method was adapted to donkey milk, because of the lower lipid content and its larger variation in donkey milk than in cow milk. The lipids in 0.7 g of the lyophilized milk samples were methylated directly using 4 mL of 0.5 M sodium methanolate plus 1.5 mL of hexane for 15 min at 50°C, and this was followed, after cooling, by the addition of 2 mL of 12 M HCl at 50°C for 15 min. Six mL of 6%  $K_2CO_3$  water solution was added after cooling. The FA methyl esters were separated as a supernatant after centrifugation and injected into a GC equipped with a flame ionization detector, separating and identifying the FA methyl esters as described by Coppa *et al.* (2015b), with the sole adaptation of the split ratio

to the lower fat content of donkey milk: a volume of 1  $\mu$ L was injected into the column at a split ratio ranging from 2.5:1 to 100:1, according to the fat content of the sample.

## *Statistics*

Statistical analyses were performed using the SPSS for Windows software package (version 17.0; SPSS Inc., Chicago, IL). The milk composition data were processed using the general linear model (GLM) of ANOVA, in which the farm was the fixed factor and the lactation stage (DIM) was the covariate. The Bonferroni test was used as the *post-hoc* test. A principal components analysis (PCA) was performed on the main FA.

## **Results**

### *Milk Gross Composition and Fat-Soluble Vitamin Content*

The fat-soluble vitamin content of the donkey milk differed significantly for all the parameters over the different farms (Table 2), except for the lactose concentration. The highest protein content was found in the milk collected on Farm 5, while the highest fat content was found in the milk from Farm 4. Only the protein content was affected by the lactation stage, with the highest protein content corresponding to the beginning of the lactation period (Table 2). However, Fischer's F for the farm effect was far higher for the farm effect than for the DIM (Table 4).

The retinol content was within the 0.89 to 4.66 $\mu$ g/100 mL range, and  $\alpha$ -tocopherol was within the 2.14 to 38.40 $\mu$ g/100 mL range. A farm effect was seen for both vitamins, with the highest levels being found in the milk on Farm 3 (Table 2).

### 193 *Milk Fatty Acid Composition*

194 The FA composition of the donkey milk differed significantly over the farms (Table 5,  
195 and supplementary Table 1, for the detailed FA profile). The milk from Farm 3 showed  
196 the highest concentrations of C18:1c9, total C18:1cis isomers, C22:5n-3,  
197 CLAc9t11 and total CLA, and the lowest concentrations of C8:0, C12:0, C14:0, total  
198 *de novo* synthesis FA, and even chain-saturated FA (ECSFA). The highest  
199 concentration of C18:3n-3, PUFA, and n-3 FA and the lowest value of the  
200 Atherogenicity and Thrombogenicity indexes were observed in the milk from Farms  
201 3, 4 and 5. The odd chain-FA (OCFA) and branched chain-FA (BCFA) concentrations  
202 were the highest in the milk from Farm 2 and the lowest in the milk from Farms 3 and  
203 6, with intermediate values in the milk from Farms 4 and 5 for BCFA. The  
204 OCFA/BCFA ratio showed the lowest value in the milk from Farm 2 and the highest  
205 in the milk from Farms 3 and 4.

206 Only a few FA were affected to a great extent by DIM. An increase in the  
207 concentrations of C14:1c9, C15:0, isoC16:0, C17:0, C18:1t11, C18:2c9t12, C18:2n-  
208 6, C18:3n-3, C22:0, C20:3n-3+C22:1c13, OCFA, PUFA, total C18:1trans isomers  
209 and n-3 FA increased with increasing DIM, whereas the concentrations of C8:0,  
210 C20:4n-6, then-6/n-3 ratio and the Trombogenicity Index decreased with increasing  
211 DIM. However, Fischer's F for those FA that showed a significant effect of both DIM  
212 and farm were far higher for the farm effect than for the DIM (Table 4).

213 The results of the PCA performed on the main FA concentrations are given in Fig.  
214 1. The PCA separated samples according to the farm in which milk was produced on  
215 both the first principal component (PC1) and the second PC (PC2) (Figure 1). The  
216 milk samples from Farm 3 were clearly separated from those of the other farms on  
217 PC1, whereas the samples from Farm 4 was separated for Farm 3 and from Farms 1,

2 and 6 on both PC1 and PC2. The samples from Farm 5 were in an intermediate position between those from Farm 4 and from Farms 1, 2, and 6, which were not separated by the PCA (Figure 1). The first principal component (PC 1, 46.4% of variance) was positively and closely correlated to ECSFA, the total *de novo* synthesis FA, the Atherogenicity index and the Trombogenicity index (correlation coefficients > 0.88), while PUFA, n-3FA and total CLA were negatively correlated to PC1 (correlation coefficients < -0.76). PC2 (33.6% of variance) was highly positively correlated with C16:0, C18:1c9 and MUFA (correlation coefficients > 0.80) and negatively correlated with n-3 FA, total *de novo* synthesis FA and PUFA (correlation coefficients < -0.53). The n-6/n-3 ratio and the OCFA/BCFA texture also made significant and positive contribution to PC2 and negative contribution to PC1, respectively (correlation coefficients > 0.51 and < -0.46).

## Discussion

### *Effect of Lactation Stage on Donkey Milk Gross Composition*

The mean protein content of milk observed in the present study is in accordance with previous data reported for donkey milk in Italy (e.g. Salimei *et al.*, 2004; Cavallarin *et al.*, 2015). The decrease in the protein content of the donkey milk during lactation is in agreement with the findings of Salimei *et al.* (2004), Giosuè *et al.* (2008), Salimei and Fantuz (2012) who reported overall values ranging from a maximum of 2.1 g/100 mL, at the beginning of lactation, to a minimum of 1.6 g/100 mL in late lactation.

### *Effect of Lactation Stage on the Fatty Acid Composition of Donkey Milk*

The effect of lactation on the FA composition of donkey milk was studied by Martemucci and D'Alessandro (2012), Gubić *et al.* (2015) and Martini *et al.* (2015). These authors highlighted an increase in concentration of long chain FA and a

decrease in concentrations of short chain FA from *de novo* synthesis in the mammary gland, with the development of the lactation stage. These results are in agreement with the significant increase observed for several long-chain FA during lactation in the present study, even if the differences found in literature in donkey milk FA composition during lactation were larger than those observed in the present study. However, the aforementioned studies followed the evolution of the FA composition of milk collected from individual animals throughout the entire lactation period in controlled condition and with a constant diet (Martemucci and D'Alessandro, 2012; Martini *et al.*, 2015). On the other hand, the effect of animal related factors, such as breed and lactation stage, are known to have a negligible effect on the FA composition of milk in dairy cows on farms, compared to animal diet (Coppa *et al.*, 2015a). The results of the present study have shown a greater effect on milk FA of the farming system, with a limited effect of the lactation stage, which is pointed out by the higher ANOVA Fisher's F coefficients for the Farm effect than for DIM.

#### *Effect of Feeding System on the Gross Composition of Milk*

To the best of our knowledge, the effect of the feeding system on donkey milk quality has never been studied before. The higher content of fat in the milk collected on Farm 3 and 4 corresponded to a higher pasture proportion in the diet than on the other farms. In addition, the hay sampled on Farm 4 in two different periods (data not shown) resulted to be of high quality, in terms of protein and ADF content. This indicates that forage quality plays an important role in the fat concentration of donkey milk.

It is well known that, in ruminants, genetics may also accounts for the difference between the protein and fat contents of milk (Shingfield *et al.*, 2013). No evidence is available in this regard for equine species. It can be speculated that the higher

content of milk protein from Farm 2 and 5 might depends on the fact that a homogenous breed is reared on these farms (Martina Franca and Ragusana, respectively), unlike the other farms, where crossbreeds animal are reared.

#### *Effect of Feeding System on the Fat-soluble vitamin content of the milk*

Little is known about the fat-soluble vitamin content in donkey milk. Gentili *et al.* (2013) and Clayer *et al.* (2014) reported the average contents of  $\alpha$ -tocopherol and retinol in donkey milk, and compared them with milk from other species. However, the variations in fat-soluble vitamins in donkey milk fed different diets have never been studied before. Álvarez *et al.* (2015) reported a concentration of retinol in milk from mares fed at pasture that was double that reported by other authors for mares fed hay (Khul *et al.*, 2012). Similarly, the amount of  $\alpha$ -tocopherol and retinol in cow milk was shown to double approximately when cows were fed at pasture instead of conserved forages (Nozière *et al.*, 2006). These provitamin carotenoids originate from  $\beta$ -carotene through enzymatic oxidative. As  $\beta$ -carotene is highly sensitive to ultraviolet light, it is degraded into forages during herbage wilting in the field, and this results in the hay having lower  $\beta$ -carotene contents than the fresh herbage (Nozière *et al.*, 2006). Thus, the higher concentrations of  $\alpha$ -tocopherol and retinol in the milk from Farm 3 than in milk from the other farms are coherent with the high proportion of fresh herbage in the donkey diet.

#### *Effect of Feeding System on the Fatty Acid Composition of the Milk*

The present results are the first evidence of the effect of feeding system on the detailed milk FA profile of donkey milk on commercial farms, as the only study available in literature, in which the FA composition of donkeys fed different diets was compared in controlled conditions, was focused on a few groups of FA (Chiofalo *et*

al., 2005). Our study points out an important influence of animal diets on the FA profile of donkey milk. The milk collected in Farm 3 showed the highest concentration of the FA that are favorable for human nutrition, such as C18:1c9, C18:3n-3, n-3 FA and PUFA, and the lowest concentration of the FA less favorable for human health, such as ECSFA, and *de novo* synthesis FA (Salimei and Santuz, 2012, Claeys *et al.*, 2014). The key factor that can explain the FA pattern of the milk from Farm 3 is related to the donkey diets, which were exclusively constituted by fresh forage from pastures. The higher concentration of C18:3n-3, compared to that in the milk from the other farms, could be derived from a direct transfer of this FA from the ingested pasture (Chiofalo *et al.*, 2005), as C18:3n-3 is the most abundant FA in fresh herbage (Coppa *et al.*, 2015b). A higher transfer of C18:3n-3 in the milk of equids than that of ruminants is allowed by the lack of biohydrogenation (Claeys *et al.*, 2014), which conversely occurs for most of the ingested long-chain PUFA in ruminants (Shingfield *et al.*, 2013). The C18:3n-3 has been shown to be a valuable indicator of pasture feeding for dairy cows (Farruggia *et al.*, 2014; Hurtaud *et al.*, 2014), and its concentration has been shown to increase with increasing fresh herbage proportions in cow diets (Coppa *et al.*, 2012). The increase in the C18:3n-3 concentration in donkey milk, with increasing proportions of fresh herbage in the diet, is also consistent with the intermediate concentration of this FA in the milk from Farms 4 and 5, which had 50 and 40% of fresh herbage in the diets, respectively.

The higher C18:3n-3, C22:3n-3 and PUFA proportions in the donkey milk for Farm 3, due to the full grazing diet, could also have partially inhibited the *de novo* synthesis process in the mammary gland (Shingfield *et al.*, 2013; Claeys *et al.*, 2014), thus resulting in lower concentrations in the milk of C8:0, C10:0, C12:0, C14:0, total *de novo synthesis* FA, and ECSFA. A lower concentration of SFA in the milk from



donkeys fed fresh herbage than those from donkeys fed hay was also observed by Chiofalo *et al.* (2005), as observed for ruminants (Shingfield *et al.*, 2013).

Small concentrations of CLAc9t11 have been observed in horse milk, but have never been detected in donkey milk before (Devle *et al.*, 2012; Medhammar *et al.*, 2012). However, the same authors reported concentrations of C18:1t11 in donkey milk. This FA is known to be the substrate for CLAc9t11 desaturation by  $\Delta^9$ -desaturase activity in the mammary gland in ruminants (Shingfield *et al.*, 2013), and to be responsible for the desaturation in the mammary gland of donkeys (Martemucci and D'Alessandro 2012), thus suggesting a possible similar origin in donkey milk. The CLAc9t11 in ruminants can also originate from dietary C18:2n-6 biohydrogenation by *Butyrivibrio* sp. bacteria, as well as C18:1t11 from C18:3n-3 (Kemp and Lander, 1984). *Butyrivibrio* sp. bacteria were also identified as main components of equine gastrointestinal compartments (Daly *et al.*, 2012; Sadet-Bourgeteau and Julliand, 2012). This would seem to suggest that a small part of ingested C18:3n-3 may have been biohydrogenated, by these bacteria to C18:1t11, which could have been desaturated to CLAc9t11 in the mammary gland. This hypothesis also seems to be supported by the higher concentrations of both C18:1t11 and CLAc9t11 in the milk of Farm 3, in which the donkeys were fed at pasture. In fact, C18:1t11 and CLAc9t11 have been identified as indicators of pasture proportion in cow diets for ruminants (Hurtaud *et al.*, 2014; Coppa *et al.*, 2012 and 2015b).

The variations in OCFA and BCFA in donkey milk, according to the feeding system, are more difficult to interpret, as little is known about the mechanism that determines their concentration. Only Devle *et al.* (2012) and Medhammar *et al.* (2012) reported the average OCFA and BCFA concentrations in donkey milk. The OCFA

341 and BCFA in the milk of ruminants are mainly derived from the lipid membrane of  
342 ruminal bacteria (Vlaemink *et al.*, 2006). Their concentration in cow milk varies  
343 according to the shift in ruminal population due to the changes in ruminal substrate,  
344 as a function of the different diets (Vlaemink *et al.*, 2006). In particular, forage-based  
345 diets favor the cellulolytic bacteria population in rumen, and determine an increase in  
346 BCFA in milk (Vlaemink *et al.*, 2006; Coppa *et al.*, 2015a). On the other hand, the  
347 substitution of hay or pasture feeding with corn silage or cereal based-concentrates,  
348 which are rich in starch, favors the ruminal population of amylolytic bacteria, with a  
349 resultant increase in the milk concentration of OCFA and of the OCFA/BCFA ratio. In  
350 addition, the concentration of BCFA in cow milk has also been negatively related to  
351 the diet protein and total FA contents (Vlaemink *et al.*, 2006), that arise from legume  
352 and oilseed supplementations. The main cellulolytic bacteria in cow rumen are  
353 *Ruminococcus flavescentis*, *R. albus*, *Fibrobacter succinogenes* and *Butyrivibrio* sp.  
354 These bacteria are also the main cellulolytic bacteria in the equine gastrointestinal  
355 compartments (Sadet-Bourgeteau and Julliand, 2012; Costa *et al.*, 2015). Similarly,  
356 *Megasphaera elsdenii* and *Streptococcus bovis*, which are among the main amylolytic  
357 bacteria of cow rumen, are also important components of the gastrointestinal flora of  
358 equines (Sadet-Bourgeteau and Julliand, 2012; Costa *et al.*, 2015). *Streptococcus*  
359 *bovis* also plays a proteolytic role in cow rumen (Vlaemink *et al.*, 2006). The changes  
360 in microbiota population in the gastrointestinal compartments of equine fed grass or  
361 concentrate diets (Daly *et al.*, 2012) are also in line with the findings observed for the  
362 ruminal population in cows (Vlaemink *et al.*, 2006), which would therefore suggest a  
363 similar regulation mechanism of the microbiota in cow rumen and equine  
364 gastrointestinal compartments. Even though the *de novo* synthesis of small amounts  
365 OCFA and BCFA cannot be excluded, as for ruminants (Vlaemink *et al.*, 2006), the

results on the milk OCFA and BCFA concentrations in donkey milk also seem to support the hypothesis of their bacterial origin in equids. In fact, the OCFA concentrations were the highest in the farms in which the donkeys were supplemented with cereal-based concentrates. The OCFA/BCFA ratio showed the lowest values in the milk from Farms 3 and 4, in which the donkeys were fed at pasture (with a higher protein content and lower fiber content than hay) and with pasture and hay, respectively, without any cereal-based concentrate. The concentrate supplementation on Farm 5, which had a slightly lower fresh herbage proportion than Farm 4, could have reduced the effect of the diet on the OCFA/BCFA ratio. Farm 2 showed the lowest OCFA/BCFA ratio, which could be explained by the presence of oilseeds in the concentrate composition.

The present research has highlighted the effect of feeding system on the composition of donkey milk, which has here been shown to be more relevant than the effect of lactation stage. Pasture feeding has been shown to improve the milk fat content and fat-soluble vitamin concentration of donkeys and to move the FA composition of the milk to a more favorable profile for human nutrition, as already observed for ruminants.

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**Table 1** *Farm characteristics obtained from on farm survey*

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6
Total donkeys <sup>1</sup> (n.)	53	63	150	60	130	48
Jennies <sup>1</sup> (n.)	44	40		40	80	32
Milking jennies <sup>1</sup> (n.)	6	12	9	6	10	10
BW of milking jennies(kg)	213	353	307	276	321	337
BCS of milking jennies	2.3	2.8	2.3	3.3	1.8	3.4
Breed	Crossbreeds	Martina Franca	Crossbreeds	Crossbreeds	Ragusana	Crossbreeds
Milking system	Automatic in milking room	Automatic in milking room	Hand milking	Automatic in cowshed	Automatic in milking room	Automatic in milking room
Milk yield (L/animal×d)	0.5	0.7	0.8	1.1	2.0	1.0
Feeding	Pasture 0%	Pasture 0%			Pasture 40%	Pasture 0%
	Hay 90%	Hay 90%	Pasture 100%	Pasture 50%	Hay 50%	Hay 100%
	Cereal mix A <sup>2</sup> 10%	Cereal mix B <sup>2</sup> 10%		Hay 50%	Cereal mix A <sup>2</sup> 10%	

<sup>1</sup>Counted during the visit.

<sup>2</sup>Cereal Mix A = 60% cereals, 30% cereal by-products, 10% legumes; Cereal mix B = 40% cereals, 40% cereal by-products, 10% legumes, 10% oilseeds.



**Table 2** *Composition and fat-soluble vitamin contents of the donkey milk on the studied farms*

Milk constituents	Farm						SEM	Effect and	
	1	2	3	4	5	6		DIM	Farm
Fat (g/100 g milk)	0.13 <sup>b</sup>	0.17 <sup>b</sup>	0.36 <sup>ab</sup>	0.65 <sup>a</sup>	0.25 <sup>b</sup>	0.26 <sup>b</sup>	0.03	NS	**
Protein (g/100 g milk)	1.76 <sup>b</sup>	1.96 <sup>a</sup>	1.84 <sup>b</sup>	1.65 <sup>b</sup>	2.03 <sup>a</sup>	1.93 <sup>b</sup>	0.04	***	***
Lactose (g/100 g milk)	7.90	7.49	6.87	6.39	7.60	6.68	0.23	NS	NS
Retinol (µg/100 mL)	0.91 <sup>b</sup>	1.36 <sup>ab</sup>	3.04 <sup>a</sup>	2.78 <sup>ab</sup>	2.84 <sup>ab</sup>	1.82 <sup>ab</sup>	0.21	NS	**
α-Tocopherol (µg/100 mL)	3.13 <sup>b</sup>	5.80 <sup>b</sup>	25.79 <sup>a</sup>	19.31 <sup>ab</sup>	8.57 <sup>b</sup>	5.58 <sup>b</sup>	2.23	NS	*

<sup>1</sup>DIM = days in milk; NS = not significant.

<sup>a,b</sup>Values within a row with different superscripts differ significantly at: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

**Table 3** Recoveries of the method used for the determination of retinol and  $\alpha$ -tocopherol in donkey milk

	Spiking level ( $\mu\text{g}/100\text{ mL}$ )	Recovery $\pm$ SD <sup>1</sup> (%)	RSD <sup>2</sup> (%)
Retinol	2.70	91.6 $\pm$ 1.67	1.82
	43.0	96.8 $\pm$ 8.51	8.80
	107	91.1 $\pm$ 4.95	5.43
	Mean of means	93.2 $\pm$ 3.16	3.39
$\alpha$ -Tocopherol	5.40	105 $\pm$ 4.25	4.02
	54.0	86.5 $\pm$ 3.04	3.52
	86.0	79.9 $\pm$ 2.55	3.19
	Mean of means	90.7 $\pm$ 13.4	14.8

<sup>1</sup>SD= Standard Deviation (no = 3 replicates)<sup>2</sup>RSD = relative standard deviation

**Table 4** Fischer's *F* for farm effect and days in milk (covariate factor) from the analysis of the variance and significant regressive coefficients of the covariate factor

Item	DIM <sup>1</sup>	Fisher's F <sup>1</sup>	
	Coefficient	DIM <sup>1</sup>	Farm
Protein (g/100 g milk)	-0.002069	6.55	22.48
Fatty acid (g/100g FA)			
C8:0	-0.006593	5.08	8.35
C14:1c9	0.000402	5.44	13.84
C15:0	0.000228	3.84	10.40
isoC16:0	0.000223	2.47	6.40
C17:0	0.000724	3.27	6.42
C18:1t11	0.000463	14.54	29.95
C18:2c9t12	0.000059	3.90	6.60
C18:3n-6	0.000114	8.50	11.10
C18:3n-3	0.026447	8.49	18.34
C22:0	0.000041	4.19	9.65
C20:3n-3+C22:1c13	0.000372	6.65	11.20
C20:4n-6	-0.000058	3.70	9.94
OCFA	0.001575	2.92	6.62
PUFA	0.02557	8.82	14.83
∑ n-3	0.028655	8.33	18.51
∑ n-6/∑ n-3	-0.000655	6.76	10.70
Trombogenicity Index	-0.000431	6.89	20.28

<sup>1</sup>DIM = days in milk; NS = not significant; FA = fatty acids; OCFA = odd chain-FA; PUFA = polyunsaturated FA; ∑ n-6 = sum of n-6 FA; ∑ n-3 = sum of n-3 FA.

† = *P* < 0.1.

**Table 5** Fatty acid composition of the donkey milk on the studied farms

Fatty acids (g/100 g FA)	Farm						SEM	Effect and significance <sup>1</sup>	
	1	2	3	4	5	6		DIM	Farm
C4:0	0.60	0.57	0.31	0.53	0.85	1.02	0.09	NS	NS
C6:0	0.38 ab	0.50 a	0.33 b	0.32 b	0.41 ab	0.33 ab	0.02	NS	**
C8:0	4.53 ab	5.25 a	3.54 b	4.04 ab	4.48 ab	4.31 ab	0.18	**	**
C10:0	9.71 ab	9.42 ab	6.44 b	8.44 ab	9.45 ab	10.01 a	0.37	NS	*
C10:1c9	1.57 ab	1.26 ab	0.75 c	1.68 a	1.04 bc	1.26 ab	0.06	NS	***
C12:0	9.20 a	7.76 a	4.88 b	8.74 a	8.30 a	9.28 a	0.35	NS	**
C12:1c9	0.18 a	0.12 b	0.07 c	0.21 a	0.11 bc	0.14 ab	0.01	NS	***
isoC14:0	0.12 a	0.11 ab	0.07 bc	0.07 bc	0.12 a	0.06 c	0.01	NS	**
C14:0	7.52 a	6.60 a	4.10 b	7.52 a	6.61 a	7.44 a	0.25	NS	***
isoC15:0	0.11 ab	0.13 a	0.06 c	0.08 bc	0.08 bc	0.08 bc	0.01	NS	***
anteisoC15:0	0.10 b	0.13 a	0.06 c	0.05 c	0.08 bc	0.06 c	0.01	NS	***
C14:1c9	0.40 ab	0.29 bc	0.19 c	0.48 a	0.26 bc	0.40 ab	0.02	*	***
C15:0	0.44 a	0.38 b	0.29 cd	0.33 bc	0.34 bc	0.25 d	0.01	†	***
isoC16:0	0.23 a	0.24 a	0.15 b	0.15 b	0.16 ab	0.16 ab	0.01	*	***
C16:0	20.72	20.50	18.99	19.00	18.76	19.25	0.29	NS	NS
C16:1c9	3.78	3.32	3.79	3.28	2.45	4.47	0.20	NS	NS
anteisoC17:0	0.22 b	0.27 a	0.17 bc	0.19 bc	0.21 b	0.15 c	0.01	NS	***
C17:0	0.35 ab	0.47 a	0.22 ab	0.22 ab	0.30 ab	0.19 b	0.02	*	*
C17:1c9	0.43 a	0.41 a	0.35 ab	0.43 a	0.28 b	0.30 b	0.01	NS	**
C18:0	1.55 bc	1.86 ab	1.90 ab	1.01 c	2.02 a	1.49 bc	0.06	NS	***
C18:1t11	0.10 b	0.09 b	0.30 a	0.14 b	0.21 ab	0.11 b	0.02	**	***
C18:1c9	17.19 ab	17.64 ab	20.74 a	12.59 b	14.70 b	16.84 ab	0.67	NS	*
C18:1c11	1.28	1.31	1.38	0.89	1.02	1.45	0.05	NS	†
C18:2c9t12	0.031 ab	0.027 b	0.047 a	0.027 b	0.034 ab	0.026 ab	0.002	*	***
C18:2n-6	6.03 b	6.87 b	8.77 a	5.43 b	9.05 a	5.48 b	0.30	NS	***
C18:3n-6	0.061 b	0.071 b	0.097 ab	0.111 a	0.076 bc	0.076 bc	0.003	**	***
C18:3n-3	9.68 b	10.69 b	17.97 a	20.54 a	14.70 ab	11.99 b	0.70	***	***
C20:1c11	0.22 ab	0.23 ab	0.25 a	0.16 b	0.18 ab	0.20 ab	0.01	NS	*
CLAc9t11	0.06 b	0.06 b	0.09 a	0.07 b	0.07 b	0.07 b	0.00	NS	**
C20:2n-6	0.16 bc	0.15 bc	0.18 ab	0.11 c	0.21 a	0.11 c	0.01	NS	***
C22:0	0.022 a	0.018 ab	0.023 a	0.016 ab	0.020 a	0.011 b	0.001	**	**
C20:3n-3+C22:1c13	0.29 b	0.32 b	0.46 a	0.47 a	0.38 ab	0.33 b	0.01	*	***
C20:4n-6	0.043 bc	0.031 c	0.066 a	0.031 c	0.064 a	0.054 ab	0.003	†	***
C24:0	0.015 b	0.018 b	0.039 a	0.010 b	0.028 ab	0.020 b	0.002	NS	***
C22:5n-3	0.077 ab	0.047 b	0.113 a	0.076 ab	0.097 ab	0.077 ab	0.006	NS	**
ECSFA	54.31 a	52.60 a	40.62 b	49.65 ab	51.00 a	53.20 ab	1.03	NS	***
OCFA	1.41 a	1.53 a	1.09 b	1.24 ab	1.20 ab	1.00 b	0.05	*	*
BCFA	0.93 ab	1.10 a	0.63 c	0.66 bc	0.77 b	0.63 c	0.04	NS	***
MUFA	26.16	25.66	29.10	20.76	21.25	26.10	0.89	NS	NS
PUFA	16.71 b	18.56 b	28.11 a	27.14 a	24.98 a	18.45 b	0.89	**	***
∑ cis18:1	18.56 ab	19.06 ab	22.25 a	13.57 b	15.81 b	18.40 ab	0.72	NS	*
∑ trans18:1	0.18 b	0.19 b	0.46 a	0.22 b	0.38 ab	0.20 b	0.02	**	***
∑ n-6	6.36 b	7.19 b	9.17 a	5.71 b	9.47 a	5.78 b	0.31	NS	***
∑ n-3	10.09 b	11.11 b	18.61 a	21.17 a	15.24 ab	12.45 b	0.71	***	***
∑ n-6/∑ n-3	0.64 a	0.65 a	0.51 ab	0.27 b	0.65 a	0.47 ab	0.02	*	***
OCFA/BCFA	1.58 ab	1.39 b	1.73 a	1.89 a	1.59 ab	1.65 ab	0.04	NS	*
∑ de novo synthesis									
FA	31.94 a	30.12 a	19.60 b	29.58 a	30.10 a	32.39 ab	1.08	NS	**
∑ CLA	0.12 b	0.12 b	0.17 a	0.13 b	0.13 b	0.11 b	0.00	NS	***

Atherogenicity Index	1.83 <sup>a</sup>	1.50 <sup>ab</sup>	0.75 <sup>d</sup>	1.11 <sup>c</sup>	1.11 <sup>c</sup>	1.61 <sup>ab</sup>	0.06	NS	***
Trombogenicity Index	0.62 <sup>a</sup>	0.57 <sup>a</sup>	0.34 <sup>c</sup>	0.35 <sup>c</sup>	0.45 <sup>bc</sup>	0.51 <sup>ab</sup>	0.02	*	***

<sup>†</sup>DIM = days in milk; NS = not significant; FA = fatty acids; ECSFA = even chain-saturated FA; OCFA = odd chain-FA; BCFA= branched chain-FA; MUFA = mono-unsaturated FA; PUFA = polyunsaturated FA; CLA = conjugated linoleic acid;  $\sum$  cis18:1= sum of cis isomers of C18:1;  $\sum$  trans18:1= sum of isomers of C18:1;  $\sum$  n-6 = sum of n-6 FA;  $\sum$  n-3 = sum of n-3 FA;  $\sum$  *de novo synthesis* FA = sum of even-chain SFA from C4:0 to C14:0;  $\sum$  CLA = sum of CLA isomers

†  $P < 0.1$ ; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

## Figure captions

Figure 1. *Principal component analysis performed on the main FA of the milk: plot of the variable<sup>1</sup> distribution and of the sample distribution.*

<sup>1</sup> ECSFA = even chain-saturated FA; OCFA/BCFA = odd chain-FA to branched chain-FA ratio; MUFA = mono-unsaturated FA; PUFA = polyunsaturated FA;  $\sum n-3$  = sum of n-3 FA;  $\sum n-6/\sum n-3$  = sum of n-6 FA to sum of n-3 FA ratio;  $\sum de\ novo\ synthesis\ FA$  = sum of even-chain SFA from C4:0 to C14:0;  $\sum CLA$  = sum of CLA isomers; AI: Atherogenicity index; TI:Thrombogenicity Index.